ECOLOGY OF CALIFORNIA ENCEPHALITIS VIRUSES ON THE DEL MAR VA PENINSULA

I. Virus Isolations from Mosquitoes

J. W. LE DUC, W. SUYEMOTO, T. J. KEEFE, J. F. BURGER, B. F. ELDRIDGE, and P. K. RUSSELL

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14. ABSTRACT

The ecology of California encephalitis viruses was studied on the Del Mar Va Peninsula. Adult mosquitoes were collected weekly from May to October of 1971 and monthly from May to October of 1972, using CDC miniature light traps with Dry Ice. Floodwater mosquitoes were assayed for virus in suckling mice or cell cultures. In 1971 over 77,000 mosquitoes were processed, resulting in 33 virus isolations. In 1972, over 106,000 were processed and 63 virus strains were recovered. Of the 1971 strains, all but one were recovered from Aedes at/anticus mosquitoes, and of the 1972 strains all but two were recovered from A. atlanticus. All A. atlanticus strains were neutralized by Keystone virus mouse hyperimmune ascitic fluid (MHAF). All other strains were recovered from A. canadensis mosquitoes. The single 1971 strain, and one strain from 1972, were neutralized by Jamestown Canyon MHAF. The remaining strain was neutralized by Keystone MHAF. The rate of virus recovery from A. at/anticus remained approximately the same, both between years and during each year studied, even during periods when large numbers of adults were emerging, suggesting that these mosquitoes had emerged infected.

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ECOLOGY OF CALIFORNIA ENCEPHALITIS VIRUSES ON THE DEL MAR VA PENINSULA

I. VIRUS ISOLATIONS FROM MOSQUITOES*

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Abstract. The ecology of California encephalitis viruses was studied on the Del Mar Va Peninsula. Adult mosquitoes were collected weekly from May to October of 1971 and monthly from May to October of 1972, using CDC miniature light traps with Dry Ice. Floodwater mosquitoes were assayed for virus in suckling mice or cell cultures. In 1971 over 77,000 mosquitoes were processed, resulting in 33 virus isolations. In 1972, over 106,000 were processed and 63 virus strains were recovered. Of the 1971 strains, all but one were recovered from *Aedes atlanticus* mosquitoes, and of the 1972 strains all but two were recovered from *A. atlanticus*. All *A. atlanticus* strains were neutralized by Keystone virus mouse hyperimmune ascitic fluid (MHAF). All other strains were recovered from *A. canadensis* mosquitoes. The single 1971 strain, and one strain from 1972, were neutralized by Jamestown Canyon MHAF. The remaining strain was neutralized by Keystone MHAF. The rate of virus recovery from *A. atlanticus* remained approximately the same, both between years and during each year studied, even during periods when large numbers of adults were emerging, suggesting that these mosquitoes had emerged infected.

Endemic foci of a number of arboviruses have been found on the Del Mar Va† Peninsula in recent years. Investigations of the ecology of Cache Valley viruses,[1-4] and eastern and western equine encephalomyelitis (EEE, WEE)[5-7] viruses have been published. During these investigations and others, evidence accumulated suggesting that members of the California encephalitis (CE) virus group also were present in the area. These data include isolations of CE virus made by the National Center for Disease Control (NCDC) while investigating an EEE outbreak,[8] acquisition of antibody against CE virus by sentinel rabbits, and antibody against CE virus in wild-caught mammals.[9] Based on these observations, investigations were initiated to ascertain which of the CE viruses are endemic, and the maintenance mechanisms of these agents in this area.

Accepted 13 April 1974.

* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council.

† A local contraction derived from Delaware, Maryland, and Virginia.

MATERIALS AND METHODS

In 1971, field investigations were centered around the Pocomoke Cypress Swamp, 5 km southwest of Pocomoke City, Worcester County, Maryland (hereafter, "the swamp"). This swamp, previously described in detail, represents a northern extension of the Dismal Swamp in Virginia, and is typical of several riparian swamps along the Pocomoke River.[5] The swamp includes two basic habitats: a disturbed swamp forest containing various hydric associations ranging from open swamp to a dry substrate of thickly matted roots and leaves, and a higher area of mixed hardwood-coniferous forest. In 1972, in addition to the swamp, studies were conducted in swamp and upland forest habitats adjacent to the Pocomoke River near Snow Hill, Maryland, in Assateague National Wildlife Refuge, Assateague Island, Virginia, and at the Wallops Island NASA Station, Virginia. Both Assateague Island and Wallops NASA Station consist mainly of salt marsh vegetation and mixed hardwood-coniferous forests. Freshwater swamps similar to the Pocomoke Cypress Swamp are not present at either of these sites. A more detailed description of Assateague Island has been presented by Buescher et al.[1]

TABLE 1

Total adult female mosquitoes collected by CDC miniature light traps with Dry Ice and processed for virus isolations. Del Mar Va Peninsula, 1971 and 1972

Mosquito species	1971		1972	
	No. collected	CE virus isolations	No. collected	CE virus isolations
Aedes atlanticus	8,548	32*	19,110	61*†
A. canadensis	42,434	1‡	63,216	2‡
A. cantator	14,559		8,599	
A. infirmatus	179		24	
A. sollicitans	136		4,856	
A. taeniorhynchus	372		2,942	
A. triseriatus	168		179	
A. vexans	1,502		832	
Anopheles bradleyi	1,189		NT§	
A. bradleyi-crucians	1,215		NT	
Culex restuans	136		NT	
Mansonia perturbans	6,859		NT	
Psorophora confinnis	NT		8	
P. ferox	417		6,894	
P. varipes	NT		1	

* All Keystone strain CE.
† 1972 isolations include 54 from the Pocomoke Cypress Swamp and 7 from Snow Hill collection sites.
‡ 1971, Jamestown Canyon strain CE; 1972, 1 Keystone; 1 Jamestown Canyon CE.
§ NT, none tested.

Adult mosquito collections were made weekly from May to October of 1971, using CDC miniature light traps supplemented with Dry Ice. Collections included sites in both the root-mat swamp and upland forest. In 1972, adult mosquito collections were made intensively for a brief period during each of the warmer months. Special day and nighttime collections were made in the swamp in 1972 during the emergence of *Aedes atlanticus* adults. Weather data were routinely monitored throughout the study. Breeding sites were recorded incidentally.

Adult mosquitoes were identified and pooled as described by Saugstad et al.[5] Based on our previous failure to detect CE virus from numerous pools of *Culiseta melanura* and *Culex salinarius* tested, and the demonstrated association of CE virus with floodwater mosquitoes,[10] only floodwater mosquitoes were processed for virus. Four milliliters of 4% bovine plasma albumin in phosphate buffered saline with antibiotics was added to each mosquito pool and ground under plastic bags with a Ten Broeck tissue grinder. Triturated pools were clarified in a refrigerated centrifuge for 10 minutes at 2,500 rpm. Paired litters of eight 1- to 3-day-old suckling mice were inoculated intracerebrally with 0.02 ml of supernate per suckling mouse. Mice were observed for 14 days, sick or dying mice were harvested, and a 10% or 20% suckling mouse brain homogenate was prepared and stored at −70° C pending identification.

In 1972, adult floodwater mosquito pools were divided equally, one-half being processed as stated above, and the other half inoculated into tube cultures of continuous BHK-21, Clone 13 cells. Mosquitoes selected for cell culture were pooled in groups of 100 females or less, ground in Wasserman tubes by adding several 6-mm glass beads and vibrated on a Vortex mixer. One ml of BME (Earle's, 20% FBS, antibiotic) medium, was added to each pool, triturated a second time, 1.0 ml of BME Earle's added again, triturated a third time, then centrifuged for 20 minutes at 1,500 rpm. An aliquot of supernate was removed, diluted, and mixed 1:2 with the same media, and 0.2 ml of this added to duplicate tube monolayers of BHK-21 cells. Remaining original suspensions were saved for reisolation. Monolayers were observed for cytopathic effect (CPE) on days 2 to 7 post inoculation. Suspect positive pools were frozen, then later passed to a second set of cell culture tubes. Passage material was clarified at 8,000 rpm for 45 minutes to eliminate virus aggregation and stored in aliquots at −70° C pending identification. Contaminated and toxic

TABLE 2

Virus isolation rates from A. atlanticus *by date of collection, Pocomoke Cypress Swamp, Maryland, 1971*

Inclusive dates	*A. atlanticus* tested	Number of isolates	Isolation rate
10 Jun	2		
11–15 Jun	55	1	1:55
16–21 Jun	100		
22–28 Jun	31	1	1:31
29 Jun–6 Jul	21		
7–11 Jul	16		
12–20 Jul	5,511	20	1:275
21–25 Jul	1,074	2	1:537
26 Jul–2 Aug	888	5	1:177
3–17 Aug	83		
18–23 Aug	112	1	1:112
24–31 Aug	25		
1– 8 Sep	48	2	1:24
9–14 Sep	131		
15–21 Sep	109		
22–28 Sep	69		
29 Sep–5 Oct	37		
6–13 Oct	4		
14–19 Oct	4		
20–30 Oct	228		
Totals	8,548	32	1:267

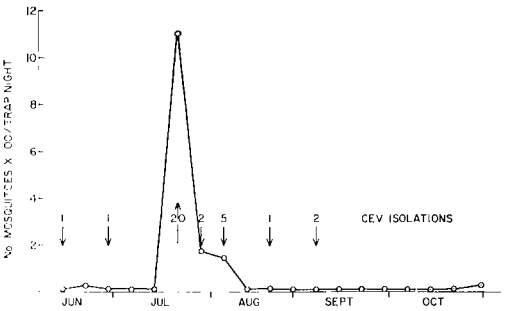

FIGURE 1. Adult female *Aedes atlanticus* per trap night as sampled by CDC miniature light traps with Dry Ice, Pocomoke Cypress Swamp, Maryland, 1971, and California encephalitis virus isolations made.

pools were retested either in cell culture or suckling mice.

Viral isolates were identified by plaque reduction neutralization tests (PRNT) on LLC-MK_2 cells.[11] Specific mouse hyperimmune ascitic fluids (MHAF) were made by the method of Brandt et al.[12] for recognized strains of the CE virus group from virus seeds supplied by the American

TABLE 3

Virus isolations from A. atlanticus *by date of collection and habitat, Pocomoke Cypress Swamp, 1971*

Inclusive dates	*A. atlanticus* collected and tested		Number of isolates		Isolation rate	
	Swamp	Uplands	Swamp	Uplands	Swamp	Uplands
10 Jun	2	0				
11–15 Jun	7	48		1		1:48
16–21 Jun	43	57				
22–28 Jun	14	17		1		1:17
29 Jun–6 Jul	11	10				
7–11 Jul	11	5				
12–20 Jul	874	4,637	2	18	1:437	1:258
21–25 Jul	256	818		2		1:409
26 Jul–2 Aug	713	175	4	1	1:178	1:175
3–17 Aug	46	37				
18–23 Aug	71	41	1		1:71	
24–31 Aug	15	10				
1– 8 Sep	24	24	1	1	1:24	1:24
9–14 Sep	25	106				
15–21 Sep	42	67				
22–28 Sep	8	61				
29 Sep–5 Oct	8	29				
6–13 Oct	1	3				
14–19 Oct	2	2				
20–30 Oct	56	172				
Totals	2,229	6,319	8	24	1:278	1:263
OVERALL TOTAL	8,548		32		1:267	

Type Culture Collection, Rockville, Maryland and Dr. Wayne Thompson, University of Wisconsin. Eight selected isolates were tested against MHAFs for Keystone, Jamestown Canyon, La Crosse, snowshoe hare, and Trivittatus viruses. These preliminary tests indicated that 6 of the 8 were strains of Keystone and 2 were strains of Jamestown Canyon; subsequently isolates were tested only against these two MHAFs.

RESULTS

During 1971, approximately 77,000 female mosquitoes, representing 13 species, were processed for virus isolation from the Pocomoke Cypress Swamp. In 1972, over 106,000 mosquitoes were processed from the combined Del Mar Va Peninsula collection sites (Table 1). From 1971 collections, 33 viruses were recovered, and from 1972 collections, 63. In 1971 only a single isolation was made from *Aedes canadensis*, the remaining being from *A. atlanticus*. Of the 63 isolations made in 1972, all but 2 were from *A. atlanticus*, the remaining 2 being from *A. canadensis*. All 1971 isolations were from material collected in the Pocomoke Cypress Swamp, while those from 1972 were from both the Swamp (56) and Snow Hill (7) sites. No virus was recovered from mosquitoes collected at either the Wallops NASA Station or Assateague Island collection sites. Virus reisolation was attempted from all positive pools, and virus was reisolated from 20 of 32 pools of *A. atlanticus* collected during 1971 and from 54 of 63 pools collected in 1972.

The field infection rate of *A. atlanticus*, including those collected early in the season and those collected during peak brood emergence, remained high. This rate ranged from as high as 1:31 from specimens collected early in the season, to 1:537 from those collected during peak emergence periods (Table 2, Fig. 1). No significant difference was detected in the 1971 isolation rate among *A. atlanticus* collected from swamp sites when compared to those collected from the adjacent upland sites (Table 3).

Many more *A. atlanticus* were tested in 1972 than in 1971. Again a high rate of virus isolation was detected, even among newly emerged adults (Table 4, Fig. 2). The overall isolation rate for 1972 approximated that seen in 1971.

All isolations made from the 1971 and 1972

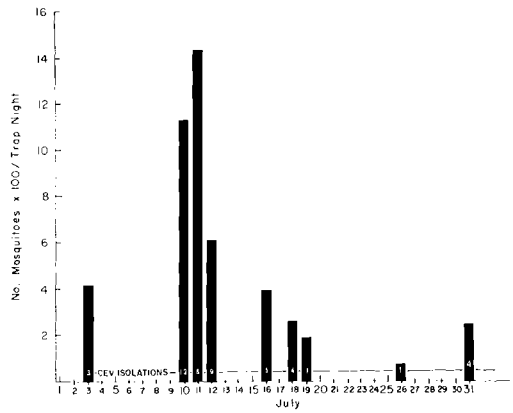

FIGURE 2. Adult female *Aedes atlanticus* per trap night as sampled by CDC miniature light traps with Dry Ice, Pocomoke Cypress Swamp, Maryland 1972, and California encephalitis virus isolations made. (Not shown are an additional isolation made on 25 November and those isolations from the Snow Hill collection sites.)

A. atlanticus pools (total of 93 strains) were neutralized by MHAF made against reference Keystone strain CE and were not neutralized by Jamestown Canyon MHAF. The neutralization test results indicate that all 93 strains from *A. atlanticus* are similar or identical to the Keystone strain.

A total of three virus isolations were made from the *A. canadensis* mosquitoes tested. The single 1971 strain and one strain recovered in 1972 were neutralized by reference Jamestown

TABLE 4

Virus isolation rates from A. atlanticus *by date of collection, Pocomoke Cypress Swamp, Maryland, 1972. Virus isolations from the Snow Hill collection site are not shown*

Collection date	*A. atlanticus* tested	Number of isolations	Isolation rate
3 July	513	3	1:171
10 July	3,393	12	1:282
11 July	4,320	8	1:540
12 July	3,643	9	1:404
16 July	1,171	5	1:234
18 July	774	4	1:193
19 July	1,150	7	1:164
26 July	431	1	1:431
31 July	2,938	4	1:734
TOTALS	18,333	53*	1:346

* One strain isolated 25 September 1972 not shown.

Canyon MHAF and were not neutralized by reference Keystone MHAF. The remaining strain was similar to the strains from *A. atlanticus* being neutralized only by Keystone MHAF.

DISCUSSION

Aedes atlanticus is a floodwater mosquito that overwinters in the egg stage and whose larvae occur in deeply shaded pools in upland forests. Eggs apparently hatch in response to flooding after heavy summer rains. In this study area a substantial rain (minimum of 3 inches in 24–48 hours) is needed to flood these pools. Such rains may occur only once a summer, so that most years only a single brood emerges. However, newly hatched larvae have been found after a second heavy rain. Since these larvae may be either from eggs laid earlier in the year, or from eggs laid during the previous year, it is impossible to state at this time whether or not *A. atlanticus* is univoltine. Adult females are abundant for a relatively few weeks, during which they readily attack a variety of mammalian and cold-blooded hosts during daytime hours.[6]

Isolations of Keystone strain CE made in 1971 and 1972 show a clear association of this virus with *A. atlanticus* mosquitoes. The remarkably high field infection rate remained roughly constant during the 2 years studied, as well as throughout each season. In both years, virus was recovered from the very first emerging females. Also, each year, as the number of adults present rapidly increased, there was no dilution detected in the rate of virus recovered. Two possibilities exist to explain these observations. First, these mosquitoes may have taken an infectious blood meal almost immediately after emergence, rapidly digested it and had virtually no extrinsic incubation period. Second, they may have emerged infected through transovarial virus transmission, as has been demonstrated by Watts et al.[13] with La Crosse strain CE. Two factors support the latter mechanism. First, engorged mosquitoes were removed before testing, thus eliminating the possibility of these isolations representing viremic blood meals. Second, the likelihood that an entire brood of mosquitoes in two successive years could rapidly acquire infections of the described magnitude is minute. We thus believe, based on the temporal relationship between *A. atlanticus* populations and isolations of Keystone strain CE made from wild caught adult females, that this virus is maintained at least in part through transovarial transmission of the agent from infected females to their progeny in this area.

The association of *A. atlanticus* mosquitoes and Keystone strain CE described here may not be unique to the Del Mar Va Peninsula. Bond et al.[14] reported several isolates of Keystone virus from *A. atlanticus-tormentor* collected in Florida, while Sudia et al.[15] reported that *A. atlanticus-tormentor* mosquito pools were the source of at least 210 Keystone virus isolations by NCDC field teams. The infection rate presented by Sudia et al.[15] of 1:368 for all *A. atlanticus-tormentor* female mosquitoes tested by NCDC is comparable to the rates included in this study and reflects collections made throughout the southeastern United States. Since our collection sites are beyond the northern range of *A. tormentor*, there probably is a close relationship between Keystone strain CE and *A. atlanticus* throughout its entire range.

The isolations of Jamestown Canyon from *A. canadensis* do little more than to establish its presence in the mid-Atlantic states.[15,16] This is not surprising, since *A. canadensis* feeds avidly on deer in this area,[6] and deer apparently are significantly involved in the maintenance of this virus in other parts of the United States.[17] The failure to isolate Jamestown Canyon virus from *A. atlanticus* and its very low infection rates detected in *A. canadensis* imply a different mechanism of virus amplification and persistence in nature from that described for Keystone virus and *A. atlanticus*.

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